

## Time Course of Plasma $T_3$ and $T_4$ Levels and Tissue Transglutaminase Activity Following Injection of Thyroid Hormones in Tadpoles

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**ABSTRACT**—Temporal changes in the plasma concentrations of  $T_3$  and  $T_4$  in premetamorphic bullfrog tadpoles (stage V-VI) were determined by radioimmunoassay following intraperitoneal injections of these hormones (0.3 nmol/g body weight), since this example of single injection is frequently used to mimic tissue changes that occur in response to the surge of circulating thyroid hormones during spontaneous metamorphic climax. At 3 hr following  $T_3$  injection, plasma  $T_3$  was 10–20 fold higher than at climax and declined to the climax level by 48 hr. The plasma  $T_4$  level was 20–50 times higher than the climax level 3 hr after  $T_4$  injection, decreasing to the climax level by 72 hr. Significant levels of plasma  $T_3$  were also detected following  $T_4$  administration, declining from 1.5 times climax level at 3 hr to climax level between 6 and 12 hr. This apparent conversion of  $T_4$  to  $T_3$  was suppressed approximately 80% by co-injection of  $T_4$  and iopanoic acid (0.3  $\mu$ mol/g body weight, i.p.), an agent that blocks the degradation of thyroid hormones. Following  $T_3$  injection, the levels of transglutaminase (TG) activity, an indicator of tissue degeneration, showed maximal increases in tail muscle (ca. 5-fold) and caudal spinal cord (ca. 6-fold) by 24 hr. In contrast, no change in TG activity was detected in hindlimb bud or lumbar spinal cord, tissues that undergo metamorphic differentiation into adult forms. Thus, despite the supraphysiological plasma levels of these hormones, changes in TG activity and other short-term tissue responses appear to mimic those occurring during spontaneous metamorphosis.

### INTRODUCTION

While premetamorphic tadpoles contain virtually undetectable levels of circulating thyroid hormones [1–4], exogenously administered triiodothyronine ( $T_3$ ) or thyroxine ( $T_4$ ) to these animals at a single dose of 0.3 nmol/g body weight (i.p.) has often been shown to induce precocious morphological and biochemical responses that mimic tissue responses to the programmed surge in endogenous levels of thyroid hormones during metamorphic climax [5–9]. These studies monitored responses in premetamorphic tissues destined for degeneration and death as well as in tissues that grow and differentiate into adult forms. While 0.3 nmol/g dose has been shown to

produce maximal tissue responses [9, 10], no reports can be found of the circulating levels of thyroid hormones generated by such intraperitoneal injections.

Because of the importance of comparing these experimentally elevated hormone levels to the known levels of  $T_3$  and  $T_4$  during spontaneous metamorphic climax [1–4], the present study was designed to examine the temporal changes in plasma  $T_3$  and  $T_4$  following single injections or co-injection of these hormones.  $T_4$ -generated  $T_3$  was also assayed because of studies suggesting that the 5'-monodeiodinase converting enzyme can be induced in premetamorphic tadpoles [11, 12]. In addition, the activity of transglutaminase (TG) was determined in spinal cord and peripheral tissues to compare the time course of a  $T_3$ -induced tissue response to the time course of plasma  $T_3$  following hormone injection. This enzyme, which is a marker of cell death-related keratinization [13], has

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been shown to increase markedly in tail cell cultures of premetamorphic tadpole treated with  $T_3$  [14].

## MATERIALS AND METHODS

### *Animals*

Bullfrog tadpoles (*Rana catesbeiana*) obtained from a local supplier were maintained at ambient temperature on a daily 12L:12D photoperiod and fed on bioled spinach every second day. The developmental stage of the tadpoles was determined on the basis of external morphology of their hindlimbs according to the criteria of Taylor and Kollros [15]. Premetamorphic tadpoles of stage V-VI were used for most of the present studies.

### *Chemicals*

Sodium salts of  $T_3$  and  $T_4$  were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Each thyroid hormone was prepared as a stock solution at 0.15 mM in 0.6 mM NaOH and stored at  $-25^\circ\text{C}$  in plastic tubes. MS-222 (ethyl *m*-aminobenzoate methane sulfonate) and N,N-dimethylcasein were also obtained from Sigma Chemical Co. RIA kits (Amerlex-M  $T_3$  and -M  $T_4$ ) for determination of  $T_3$  and  $T_4$  and NCS tissue solubilizer were purchased from Amersham International, plc (Buckinghamshire, England). [ $^3\text{H}$ ]Putrescine (1110 GBq/mmol) was obtained from Dupont-New England Nuclear (Wilmington, DE, USA). Iopanoic acid was purchased from Tokyo Kasei Chemical Co. (Tokyo, Japan). All other chemicals used were of analytical grade from Nagoya-Katayama Chemical Co. (Nagoya, Japan).

### *Treatment of tadpoles*

Premetamorphic tadpoles (stage V-VI), which had been food-deprived for two days, were anesthetized by immersion in 0.05% MS-222 and injected between 8:00 and 10:00 a.m. with 0.3 nmol/g body weight of  $T_3$  or  $T_4$  (2  $\mu\text{l}$ /g body weight stock solution), with combinations of the hormones or with 1.2 nmol of NaOH vehicle/g body weight. At varying periods following hormone injection, a 200  $\mu\text{l}$  blood sample was collected from the tadpole heart using a heparinized

syringe, and was centrifuged to obtain the plasma. Plasma samples from untreated premetamorphic tadpoles and from tadpoles at metamorphic climax stages were also prepared, and were stored at  $-70^\circ\text{C}$  prior to radioimmunoassay.

### *RIA procedure*

In order to adapt the kit for human thyroid hormone levels to tadpole plasma samples, the following modifications were made: The incubation conditions were changed from 1 hr at  $37^\circ\text{C}$  for  $T_3$  and 45 min at  $18-28^\circ\text{C}$  for  $T_4$  in human plasma to 5 hr at  $18-20^\circ\text{C}$  for tadpole plasma. Standard solutions were prepared by diluting  $T_3$  or  $T_4$  stock solutions with plasma from untreated premetamorphic tadpoles to yield final concentrations of 0.2–4.0 ng/ml of  $T_3$  or 2–100 ng/ml of  $T_4$ . Triplicate 50  $\mu\text{l}$  aliquots of tadpole plasma were used for each assay. All other RIA procedures for hormone measurement in tadpole plasma were identical with those for human plasma samples.

### *Transglutaminase (TG) assay*

TG activity in dissected spinal cord, hindlimb bud and tail muscle from premetamorphic tadpoles was assayed by following the incorporation of tritiated putrescine into dimethylcasein [16, 17]. The reaction mixture contained 1.3 mM  $\text{CaCl}_2$ , 10 mM dithiothreitol, 5 mg/ml dimethylcasein, and 0.4 mM (18 KBq) [ $^3\text{H}$ ]putrescine in 50 mM Tris-HCl buffer at pH 7.4. Tissue homogenates samples, containing 100–150  $\mu\text{g}$  protein, were added to 0.7 ml of the reaction mixture and incubated aerobically for 20 min at  $22^\circ\text{C}$ . The reaction was terminated by the addition of 0.3 ml of 20% trichloroacetic acid (TCA). After centrifugation of samples, TCA-insoluble materials were solubilized by NCS and added to the scintillation cocktail solution. Radioactivity in the samples was determined using a liquid scintillation spectrophotometer (Packard Tricarb 1500). Protein content in the samples was assayed by the method of Bradford [18] with bovine serum albumin as a standard.

## RESULTS

*Thyroid hormone levels in plasma of premetamorphic and climax stage tadpoles*

Neither T<sub>3</sub> nor T<sub>4</sub> was detectable in the plasma of untreated tadpoles at stage V-VI, confirming previous findings [1-4]. When known amounts of T<sub>3</sub> or T<sub>4</sub> were added to the plasma of these untreated premetamorphic tadpoles, recovery was greater than 90% (Table 1). Further, RIA of T<sub>3</sub> and T<sub>4</sub> in human plasma samples, diluted 1:4 with tadpole plasma and assayed by the procedure modified for amphibian samples, also yielded values greater than 90% of expected. RIA of plasma samples from tadpoles at metamorphic climax (stage XXI-XXII), by contrast, revealed high circulating levels of both hormones, comparable to values previously reported, with a T<sub>3</sub>:T<sub>4</sub> ratio of approximately 1:6 (Table 1).

*Time course of plasma T<sub>3</sub> and T<sub>4</sub> levels following hormone injection in premetamorphic tadpoles*

At 3 hr following i.p. injection of T<sub>3</sub> (0.3 nmol/g body weight) in stage V-VI tadpoles (the earliest time point examined), plasma T<sub>3</sub> levels were 10-20 fold higher than those achieved during spontaneous metamorphosis (climax values are shown as the horizontal shaded area in Fig. 1). Plasma levels declined to climax levels by 48 hr. When T<sub>3</sub> and T<sub>4</sub> were co-injected at a 1:1 ratio, plasma T<sub>3</sub>

levels declined over a similar time course at only slightly higher levels than that seen following injection of T<sub>3</sub> alone. In contrast, following co-injection of T<sub>3</sub> and T<sub>4</sub> at a 1:6 mole ratio, plasma T<sub>3</sub> levels showed a markedly slower rate of decline, suggesting *in vivo* conversion of a major portion of T<sub>4</sub> to T<sub>3</sub>. From 48 hr through 96 hr post injection, plasma T<sub>3</sub> levels were at least 6-fold higher than corresponding levels following injection of T<sub>3</sub> alone (Fig. 1).

At 3 hr following T<sub>4</sub> injection in stage V-VI

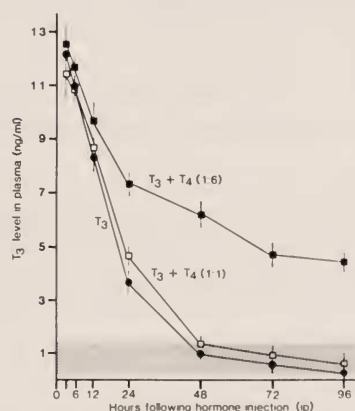


FIG. 1. Time course of T<sub>3</sub> levels in plasma of tadpoles at stage V-VI following injection of T<sub>3</sub> (0.3 nmol/g body weight, i.p.) or co-injection of T<sub>3</sub> and T<sub>4</sub> at a mole ratio of 1:1 or 1:6. Each point represents the mean of at least four experiments; vertical bars indicate SD. The horizontal shaded area indicates the range of climax levels of T<sub>3</sub> as shown in TABLE 1.

TABLE 1. Determination of thyroid hormone levels in tadpole plasma by radioimmunoassay

	T <sub>3</sub> level (ng/ml)	Recovery (%)	T <sub>4</sub> level (ng/ml)	Recovery (%)
Premetamorphic (stage V-VI)	n.d. (3)		n.d. (3)	
+ T <sub>3</sub> (1 ng/ml)	0.94 ± 0.09 (4)	94	—	
+ T <sub>4</sub> (10 ng/ml)	—		9 ± 2 (4)	90
+ human plasma <sup>a</sup>	0.74 ± 0.11 (3)	95	61 ± 4 (3)	92
Climax (stage XXI-XXII)	0.51 ± 0.12 (4)		3 ± 1 (4)	
	0.14-1.4 <sup>b</sup>		1-6 <sup>b</sup>	

Values are given as mean ± SD with number of experiments in parentheses.

n.d.: not detectable.

<sup>a</sup> Dialyzed human plasma sample containing known amounts of T<sub>3</sub> (3.90 ng/ml) and T<sub>4</sub> (330 ng/ml) was diluted 1:4 with premetamorphic tadpole plasma prior to radioimmunoassay.

<sup>b</sup> Data from Miyauchi *et al.* [1], Regard *et al.* [2] and Suzuki and Suzuki [4].

tadpoles, plasma levels of  $T_4$  reached 20–100 times higher than the peak levels of  $T_4$  achieved during spontaneous metamorphosis (compare values to shaded horizontal region in Fig. 2a). Plasma levels approached peak levels of climax by 72 hr. Apparent *in vivo* conversion of  $T_4$  to  $T_3$  was again detected, following  $T_4$  injection (Fig. 2b). For example, the 0.8 ng/ml level of  $T_3$  found 24 hr post  $T_4$  injection is comparable to the differential seen in Fig. 1 between the 24 hr  $T_3$  value following co-injection of  $T_3$  and  $T_4$  (1:1) and the value following injection of  $T_3$  alone. The appearance of  $T_3$  following  $T_4$  injection was suppressed by approximately 80% when  $T_4$  was co-injected with iopanoic acid (0.3  $\mu$ mol/g body weight), an agent that inhibits the degradation of thyroid hormones [19] (Fig. 2b).

#### Effect of $T_3$ injection on transglutaminase (TG) activity in spinal cord, hindlimb bud and tail muscle

TG activity was assayed in spinal cord and its

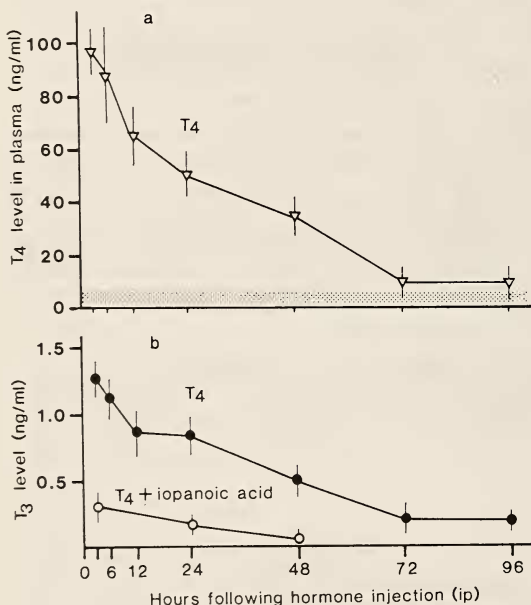


FIG. 2. Time course of  $T_4$  (a) or  $T_3$  (b) levels in plasma of tadpoles at stage V-VI following injection of  $T_4$  (0.3 nmol/g body weight, i.p.). Effect of co-injection of  $T_4$  and iopanoic acid (0.3  $\mu$ mol/g body weight, i.p.) on  $T_3$  levels is also shown in (b). Each point represents mean of at least four experiments; vertical bars indicate SD. The horizontal shaded area indicates the range of climax levels of  $T_4$  as shown in TABLE 1.

functionally related tissues, hindlimb and tail, to examine the time course of a  $T_3$ -induced tissue response. In control, vehicle-injected, premetamorphic tadpoles, TG activity was considerably lower in caudal than in lumbar spinal cord and was also lower in tail muscle than in hindlimb muscle (Table 2). Injection of  $T_3$  markedly increased the enzyme activity in caudal spinal cord and tail muscle, tissues destined for degeneration and resorption (Fig. 3, Table 2); maximal increases occurred in each tissue by 24–48 hr. Under these same conditions, TG activity in lumbar cord and hindlimb muscle, tissues destined for growth and differentiation, was unchanged.

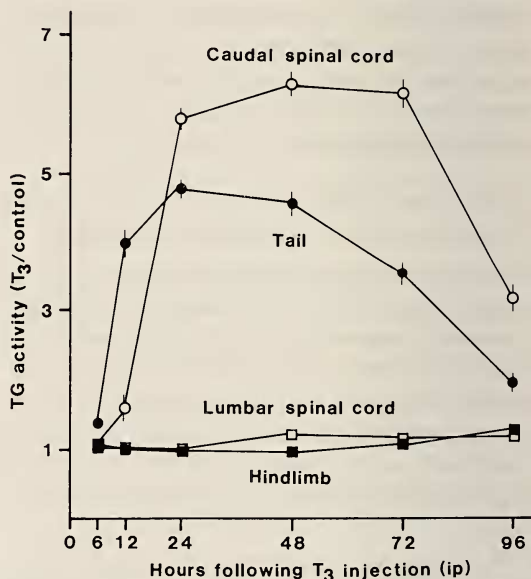


FIG. 3. Time course of  $T_3$ -induced transglutaminase (TG) activity in spinal cord, hindlimb, and tail muscle. Developmental stage of tadpole and injection protocol were similar to those described in TABLE 2. Each point represents mean of at least four experiments; vertical bars indicate SD.

## DISCUSSION

Although premetamorphic tadpoles have virtually undetectable circulating levels of thyroid hormones, a finding confirmed in the present study, the ready responses of many of their tissues to exogenous  $T_3$  and  $T_4$  have often been used as models of events at metamorphic climax [20, 21]. As an example,  $T_3$ -induced increases in activity of

TABLE 2. Effect of administered T<sub>3</sub> on transglutaminase (TG) activity in lumbar and caudal spinal cord, hindlimb bud and tail muscle of tadpoles at stage V-VI

	TG activity (nmol/mg protein/h) T <sub>3</sub>	control (NaOH)	Ratio (T <sub>3</sub> /control)
Spinal cord:			
lumbar	3.57 ± 0.60 (5)	3.09 ± 0.69 (5)	1.2
caudal	0.57 ± 0.09 (5)	0.09 ± 0.03 (5)	6.3 <sup>a</sup>
Hindlimb muscle	4.50 ± 0.78 (4)	4.71 ± 0.90 (4)	1.0
Tail muscle	5.40 ± 1.14 (7)	1.17 ± 0.24 (7)	4.6 <sup>a</sup>

T<sub>3</sub> (0.3 nmol/g body weight, i.p.) or NaOH (1.2 nmol/g body weight, i.p.) vehicle was injected into food-deprived premetamorphic tadpoles at stage V-VI. After 48 hr, lumbar and caudal spinal cord and hindlimb and tail muscle were dissected and assayed for TG activity. Values are given as mean ± SD with number of animals in parentheses.

<sup>a</sup> Ratios were significantly increased above 1.0 ( $p < 0.001$ ).

transglutaminase were detected in tail muscle and in caudal spinal cord (Table 2, Fig. 3), in agreement with previous findings that a T<sub>3</sub>-related increase in this enzyme is a harbinger of metamorphosis-related cell death [14]. Further, the 24–48 hr period required for peak increases in TG activity was similar to the time required to achieve maximal T<sub>3</sub> or T<sub>4</sub> injection-induced decreases in protein synthesis in tail muscle and tail dorsal root ganglia [6, 9]. The tissue specificity of this T<sub>3</sub> response was indicated by the lack of change in TG activity in hindlimb bud or lumbar spinal cord. These tissues, in which protein synthesis increases at 24–48 hr post T<sub>3</sub> injection [8, 9], contain a predominance of cells destined for growth and differentiation. Thus, by the time the peak responses of tissue are reached in this single-injection protocol, the premetamorphic tadpoles have been exposed to circulating levels of T<sub>3</sub> and T<sub>4</sub> as much as 10 to 100 times greater than the maximal circulating levels achieved during the endogenous surge of these hormones at metamorphic climax. The implications of these high levels of plasma thyroid hormones must be considered when evaluating the physiological relevance of the premetamorphic model system.

#### *Implications of supraphysiological levels of plasma T<sub>3</sub> and T<sub>4</sub>*

Despite the relatively high circulating levels of thyroid hormone generated by hormone injection, the initial tissue responses were not markedly different from those occurring during spontaneous

metamorphic climax. For example, the magnitude of T<sub>3</sub>-induced increase in [<sup>3</sup>H]leucine incorporation in stage V-VI lumbar dorsal root ganglia was only slightly higher than the rise in [<sup>3</sup>H]leucine incorporation during spontaneous climax [9]. Similarly, the magnitude of T<sub>3</sub>-induced decrease in [<sup>3</sup>H]leucine incorporation in premetamorphic tail [6, 9] was comparable to the decrease in incorporation during climax [22]. These similarities in tissue responses during induced and spontaneous metamorphosis are not surprising in light of evidence that T<sub>3</sub> receptors in tadpole are essentially saturated at thyroid hormone levels attained during spontaneous metamorphic climax [23]. Thus, even though plasma T<sub>3</sub> and T<sub>4</sub> levels following hormone injection were markedly higher than those achieved at climax, a greater tissue response would not be expected.

It is also possible that supramaximal levels of circulating thyroid hormones are required to produce maximal tissue responses in the premetamorphic tadpoles since T<sub>3</sub> and T<sub>4</sub> may not comprise a sufficient endocrine model of metamorphic climax. Circulating levels of corticosterone and aldosterone, for example, also sharply increase at the onset of climax [24–26]. Since both of these corticosteroids accelerate T<sub>4</sub>-induced shrinkage of isolated tail segments [27] and enhance T<sub>3</sub> binding to tail fin nuclei [28], it may be that maximal tissue responses mimicking those at metamorphic climax can be achieved in premetamorphic tadpoles by co-injecting lower doses of T<sub>3</sub> or T<sub>4</sub> with corticosteroids.

### Implications of $T_4$ conversion to $T_3$

The rapid appearance of  $T_3$  in plasma following i.p. injection of  $T_4$  may result from a combination of factors. Although the converting enzyme, 5'-deiodinase (5'D), is not detectable in serum of premetamorphic tadpoles [11], the enzyme is present at low levels in gut [12]. Thus, i.p.-injected  $T_4$  could be converted to  $T_3$  prior to its absorption into the circulation. The present results are also consistent with previous findings that 5'D can be induced in serum of premetamorphic tadpoles by endogenous thyroid hormone [11, 29]. But, whether  $T_3$  synthesis occurred via constitutive or induced 5'D, it is of interest that resulting circulating level of  $T_3$  remained within the range of that occurring during spontaneous metamorphic climax (compare Fig. 2b with Table 1). Since the climax-mimicking level of  $T_3$  was maintained despite concomitant  $T_4$  levels as much as 20–100 times greater than those at metamorphic climax, it may be that the same mechanisms that regulate climax levels of circulating  $T_3$  are at play when metamorphic change is induced by exogenous  $T_4$ .

### Implications for the physiological relevance of the premetamorphic model

The present results reveal that the injection of a single dose of 0.3 nmol  $T_3$  or  $T_4$ /g body weight in premetamorphic tadpoles, which has been frequently utilized to model events at metamorphic climax, subjects the animals for several days to supraphysiological levels of the hormones. Despite this circumstance, and the probable absence of a variety of other hormones that normally circulate at metamorphic climax [30], the model appears to reproduce the climax-related changes in 5'D and 5-deiodinase activity [12, 19] and to mimic, at least for short-term studies, many tissue responses associated with later stage tadpoles [9, 20].

The present findings stress the values of assaying plasma levels of thyroid hormones when exploring models of metamorphic events. For example, it will be important to compare the time course of plasma levels achieved following hormone injection with those resulting from hormone administration by immersion [23] and by pellet implants

[31], approaches that produce more gradual rises in circulating hormone levels. Finally, the use of multiple radioimmunoassays should be of value for creating, in premetamorphic tadpoles, a multihormonal milieu that more closely simulates the endocrine environment during metamorphic climax.

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